

New Classes of Environmental Tumor Promoters: Indole Alkaloids and Polyacetates

by Hirota Fujiki,* Takashi Sugimura* and Richard E. Moore†

Tumor promoters are known to induce ODC activity in mouse skin and that this induction can be inhibited by the application of 13-*cis*-retinoic acid. These two properties of tumor promoters were utilized for screening new tumor promoters in our environment. Two new classes of tumor promoters are presented: indole alkaloids (teleocidin and lyngbyatoxin A) and polyacetates (aplysiatoxin and debromoaplysiatoxin).

Teleocidin from streptomyces and lyngbyatoxin A, from the blue-green alga, *Lyngbya majuscula*, were able to induce ODC activity in mouse skin and showed various biological activities similar to those of TPA. Teleocidin and lyngbyatoxin A are indole alkaloids. Their tumor promoting activities became apparent in the mouse skin through a two-stage carcinogenicity test. The tumor incidence of the group treated with DMBA plus teleocidin was 100% at week 30, which was similar to that of the group given DMBA and TPA. The *in vivo* carcinogenicity test with lyngbyatoxin A is still underway. The tumor incidence of the group treated with DMBA plus lyngbyatoxin A is 80% at week 21.

A second new class of tumor promoter is polyacetate. Aplysiatoxin and debromoaplysiatoxin were isolated from another variety of blue-green alga. Aplysiatoxin and debromoaplysiatoxin induce ODC to the same degree of potency. However, aplysiatoxin induced various membrane effects, such as adhesion of cells and turnover of phospholipid with a similar concentration of TPA, teleocidin and lyngbyatoxin A. On the other hand, debromoaplysiatoxin required an amount almost 100 times greater to achieve the same effects.

We are convinced of the possibility that various classes of tumor promoters exist in our environment.

Introduction

An extensive search for mutagens and carcinogens in the human environment was undertaken by the National Cancer Center Research Institute in Tokyo (1). Several potent mutagens and carcinogens were found and identified for the first time in food and in pyrolysis products of various amino acids (2). These findings supported the proposal, made on the basis of statistical findings, that most human cancers are caused by environmental factors (3, 4). With this in mind, we began screening for new tumor promoters in the environment.

In screening for tumor promoters, we used the following four-step test; first, a test of irritancy on

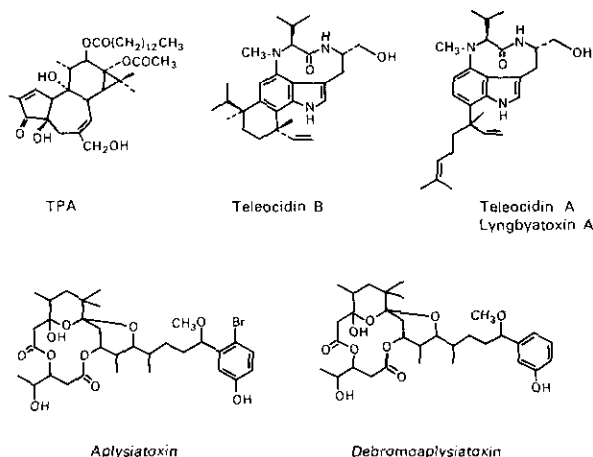
mouse ear; second, a test on induction of ornithine decarboxylase (ODC) activity in mouse skin; third a test of adhesion with human promyelocytic leukemia cells (HL-60); fourth, an *in vivo* carcinogenicity test.

We tested about 270 compounds on mouse ear. These compounds included various pure and impure compounds thought to be irritants. Of these, 43 materials caused significant reddening of mouse ear, 15 of these 43 compounds induced ODC activity with the same potency as TPA, and five of the 15 compounds caused cell adhesion of HL-60 cells. Up to now, three of five compounds have been proven to be potent promoters in an *in vivo* carcinogenicity test. We have now found five possible new tumor promoters, which are related to indole alkaloids and polyacetates. Teleocidin, dihydroteleocidin B, which was obtained by catalytic hydrogenation of teleocidin B and lyngbyatoxin A are indole alka-

*National Cancer Center Research Institute, Tsukiji 5-1-1, Chuo-ku, Tokyo 104, Japan.

†Department of Chemistry, University of Hawaii, Honolulu, Hawaii 96822.

loids. Aplysiatoxin and debromoaplysiatoxin are polyacetates.



This paper describes the various biological activities, including *in vivo* tumor-promoting activities of these five new compounds.

Methods

Irritant Test on Mouse Ear

Compounds were tested on the ear of 8-week-old female CD-1 mice. Irritancy on mouse ear was estimated 24 hr after administration of the compounds. The ID_{50}^{24} value is the amount that causes reddening of the ears of 50% of the mice (5).

Induction of ODC Activity

ODC activity was determined 4 hr after application of the compound, to the skin of the back of 8-week-old female CD-1 mice (6). The assay procedure used was essentially that of O'Brien (7, 8). Inhibition of ODC induction by 13-*cis*-retinoic acid was examined by the procedure of Verma and Boutwell (9).

In Vitro Test of Adhesion of HL-60 Cells

Adhesion of HL-60 cells was tested as described elsewhere (6, 10).

In Vivo Carcinogenicity Test

Carcinogenesis was initiated with 100 μ g of DMBA in the skin of 8-week-old female CD-1 mice. Promotion was carried out by administration 1 week later 2.5 μ g of teleocidin, 2.5 μ g of dihydroteleocidin B or 3.0 μ g of lyngbyatoxin A twice weekly. The numbers of tumors 1 mm or more in diameter were counted weekly. Tumor-promoting activity

was expressed as the percentage of tumor-bearing mice; i.e., the number of surviving mice bearing at least one skin tumor per number of survivors \times 100 (11, 12). As a positive control, 2.5 μ g of TPA was applied to the skin twice weekly.

Various *in Vitro* Biological Tests

Induction of terminal differentiation of HL-60 cells is associated with increased phagocytosis (13). Aggregation of human lymphoblastoid cells (NL-3) transformed by Epstein-Barr virus was estimated as described previously (14). Inhibition of terminal differentiation of Friend erythroleukemia cells induced by dimethyl sulfoxide was followed by measuring benzidine-reactive cells (6, 10). Inhibition of specific binding of 3 H-PDBu to rat embryo fibroblast cell monolayer cultures (FRE-8D) was observed (15). Indole alkaloids and TPA had similar effects on other biological parameters. References to the methods used are cited in Table 3 below.

Transplacental Initiation and Postnatal Promotion

Initiation was carried out by intragastric intubation of 60 mg of DMBA/kg body weight into pregnant mice on day 10 or 17 of pregnancy (16). For promotion, 2.5 μ g of teleocidin was applied twice weekly to the skin of the back of the offspring from week 8 after birth.

Materials

Teleocidin was isolated from mycelia of *Streptomyces mediocidicus* by Takashima (17). Recently we found that teleocidin is a mixture of teleocidin A, teleocidin B, and their isomers (18). Dihydroteleocidin B was obtained by catalytic hydrogenation of the vinyl group of teleocidin B (19). Teleocidin A has the same chemical structure as lyngbyatoxin A (20). Lyngbyatoxin A was isolated from a blue-green alga, *Lyngbya majuscula* (21). Aplysiatoxin and debromoaplysiatoxin, a debrominated form of aplysiatoxin, were isolated from another variety of blue-green alga, *Lyngbya majuscula* (22, 23).

Results

Irritant Test

Three indole alkaloids, teleocidin, dihydroteleocidin B, and lyngbyatoxin A, and two polyacetates, aplysiatoxin and debromoaplysiatoxin, are potent skin irritants (Table 1). The effective concentrations of these five compounds in the irritant test were very similar to that of TPA. The chemical struc-

Table 1. Comparison of the effects of indole alkaloids and polyacetates with those of TPA.

	TPA	Telocidin	Dihydro-teleocidin B	Lyngbyatoxin A	Aplysatoxin	Debromoaplysiatoxin
Irritant test						
ID ₅₀ , nmole/ear ^a	0.016	0.008	0.017	0.011	0.005	0.005
ODC assay						
Induction, nmoles CO ₂ /5.0 μ g compounds ^b	1.45	1.89	1.55	2.05	3.62 ^c	2.97 ^c
Inhibition by retinoic acid, % ^d	81.9	70.6	78.7	65.5	89.7	74.1
Tumor-promoting activity						
Maximal tumor incidence, %	100	100	90	80 ^e		

^a Results are means of six animals.^b Results are means of two animals.^c Induction was estimated as the amount per 2.5 μ g of compound.^d Results are means of two animals.^e Tumor incidence in week 21 is shown.

tures of these compounds are entirely different from that of phorbol ester.

Induction of ODC Activity

The above five compounds induced ODC activity in mouse skin. The effects of these compounds on ODC induction were all apparently inhibited by 13-*cis*-retinoic acid (Table 1). All five compounds gave bell-shaped dose-response curves, and the doses for maximum activity were in a narrow range, higher doses having cytotoxic effects. However, induction with TPA increased steadily with doses of up to 1.0 mg as previously reported (10).

In Vivo Carcinogenicity Test

The tumor incidences in groups treated with DMBA plus teleocidin, DMBA plus dihydroteleocidin B and DMBA plus lyngbyatoxin A are summarized in Table 1. The increases in tumor incidences in groups treated with DMBA plus teleocidin and DMBA plus TPA are shown in Figure 1. Both groups showed 100% tumor incidence by week 24. No tumors were observed in the groups treated with teleocidin alone or with a single application of DMBA, and only one mouse in the group treated with TPA alone had a tumor, which was identified histologically as a papilloma. The tumors in the two groups were examined histologically and classified into two types: squamous cell carcinomas and papillomas. The percentage incidences of squamous cell carcinomas and papillomas in the two groups were almost identical. These results are comparable with those on a group given DMBA plus dihydroteleocidin B reported previously (10, 24). Thus teleocidin and dihydroteleocidin B are as effective as TPA in the promotion of malignancy.

An *in vivo* carcinogenicity test with lyngbyatoxin

A is still underway, but the tumor incidence in the group treated with DMBA plus lyngbyatoxin A was 80% in week 21. Carcinogenicity tests have not yet been done on aplysiatoxin and debromoaplysiatoxin.

Various Biological Activities

The three indole alkaloids all induced both adhesion and differentiation of human promyelocytic leukemia cells (HL-60), induced aggregation of human lymphoblastoid cells (NL-3), and inhibited differentiation of Friend erythroleukemia cells with potencies equivalent to those of TPA. The doses of these compounds necessary for these four activities

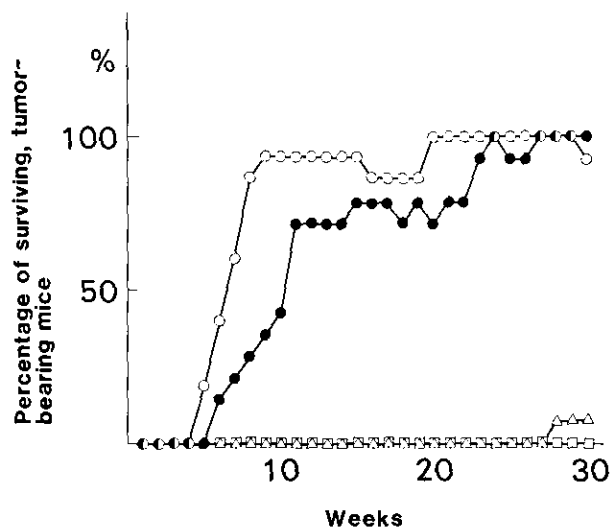


FIGURE 1. Tumor incidences in mice treated with 100 μ g DMBA followed by 2.5 μ g teleocidin and (O) 100 μ g DMBA followed by 2.5 μ g TPA. One animal in the group treated with 2.5 μ g TPA alone had a tumor (Δ). No tumors were observed in groups treated with 2.5 μ g teleocidin alone, or 100 μ g DMBA alone (\square). Results are from groups consisting of 15 animals each.

were exactly the same as those of TPA (6, 10) (Table 2).

It is interesting that various membrane effects, such as adhesion, differentiation of HL-60 cells, and aggregation of NL-3 cells, were induced by similar concentrations of aplysiatoxin and TPA (25).

Since indole alkaloids are structurally unrelated to TPA, it was of interest to determine whether they have similar effects to TPA on cell membrane receptors. We found that TPA, teleocidin and lyngbyatoxin A all inhibit the specific binding of ^3H -PDBu (15, 26) (Table 2). From this finding we conclude that these compounds may act via the same receptor system. Recently, Huberman (27) isolated a TPA-resistant mutant of HL-60 cells, and found that this mutant showed both TPA resistance and teleocidin resistance. Similar coincidence has

been observed with Friend erythroleukemia cells (28) and nematodes (29) (Table 3).

Transplacental Initiation and Postnatal Promotion

An experiment on transplacental initiation and postnatal promotion was performed in mice. The tumor incidence in the offspring of the group treated with DMBA on day 17 of gestation with subsequent promotion with teleocidin was 47% in week 23. The offspring of mice treated with DMBA on the 10th day of gestation with subsequent promotion with teleocidin showed 10% tumor incidence. Those of mice treated with teleocidin but not DMBA did not have any tumors.

Table 2. Comparison of various biological effects of indole alkaloids and polyacetates with those of TPA.

	TPA	Teleocidin	Dihydro-teleocidin B	Lyngbyatoxin A	Aplysia-toxin	Debromo-aplysiatoxin
HL-60 cells						
50% adhesion, ng/ml	1.5	4.0	0.3	7.0	2.0	180
ED ₅₀ of phagocytosis, ng/ml	2.5	3.6	1.4	2.5	1.7	100
Aggregation of NL-3 cells						
ED ₅₀ , ng/ml	11.2	3.1	6.5	2.4	2.1	180
Inhibition of differentiation of Friend erythroleukemia cells						
ED ₅₀ , ng/ml	1.0	2.0	0.2	0.4		150
Inhibition of specific binding of ^3H -PDBu						
ED ₅₀ , ng/ml	3.0	5.0		8		48

Table 3. Similar effects of TPA and indole alkaloid.

Effect	Reference
<i>In Vivo</i> effect	
Induction of dark keratinocytes in mouse skin	(35)
Induction of hyperplasia and hyperkeratosis in mouse skin	(10)
Membrane effect	
Increase in 2-deoxyglucose uptake	(15)
Release of arachidonic acid	(15)
Formation of prostaglandins	(36)
Release of choline	
Inhibition of epidermal growth factor binding	(15)
Production of superoxide anion radical ($\text{O}_2^{\cdot-}$) in human polymorphonuclear leukocytes	(37)
Adaptation to allosteric site in phospholipase A_2	(38)
Differentiation Inhibition or Induction	
Inhibition of induced melanogenesis in B16 melanoma cells	(39)
Inhibition of induced myogenesis in human myoblasts	(40)
Amelanotic effect and morphological change of reconstituted cells	(39)
Coincidence with TPA resistance in Friend erythroleukemia cells	(41)
Transformation, adenovirus and EB virus	
Increase in transformed foci after treatment with chemical carcinogen	(42)
Enhancement of colony formation of Adenovirus-infected lymphocytes	(40)
Enhancement of colony formation of EBV-infected cord blood lymphocytes	(14)
Enhancement of early antigen and viral capsid antigen production	(43)
Others	
Induction of abnormal movements of a nematode (<i>Caenorhabditis elegans</i>)	(44)
Coincidence with TPA-resistance of mutants of a nematode (<i>C. elegans</i>)	(44)

Actions of Aplysiatoxin and Debromoaplysiatoxin

A 100 times greater dose of debromoaplysiatoxin was required to achieve the same effect (10, 13). This means that bromination of the phenol groups in the molecule of debromoaplysiatoxin greatly enhanced the effect on the cell membrane (Table 2).

Discussion

This paper described new tumor promoters which were detected by our four-step screening method for tumor promoters in the environment; i.e., an irritant test on mouse ear, a test of ODC induction in mouse skin, a test of adhesion with HL-60 cells and an *in vivo* carcinogenicity test. The irritant test is a sensitive and practical method as a first screening test. It can be used for various kinds of compounds from a wide variety of sources and does not usually require a large amount of material. The ID_{50} values of newly found tumor promoters are in the range of 5-10 ng. Since ODC induction in mouse skin appears to be an essential phenotypic change for tumor promotion (30), ODC assay is useful as a second screening test. However, several compounds were found to show similar effects to TPA in the test of ODC induction, but were not tumor promoters or were only weak promoters in mouse skin (31-34). To distinguish these nonpromoters, we introduced a third test on cell membrane effects, even though the mechanistic relation between tumor promotion and membrane phenomena needs clarification.

Experiments on transplacental initiation in pregnant mice and postnatal promotion by teleocidin as well as TPA on the skin of the back of the offspring have opened up a new concept of tumor promotion.

It is now known that the human environment contains a number of mutagens and carcinogens, and it is important to find out how to protect humans from contact with these environmental tumor promoters, since this is crucial for preventing human cancer.

Carcinogenicity tests on aplysiatoxin and debromoaplysiatoxin will provide critical information on the utility of the third test. We are certain that various classes of tumor promoters are present in our environment and that other tumor promoters will be found by the four-step screening.

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